# TGF-β1-induced cell migration in pancreatic carcinoma cells is RAC1 and NOX4-dependent and requires RAC1 and NOX4-dependent activation of p38 MAPK

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**Abstract.** Transforming growth factor (TGF)-β promotes epithelial-mesenchymal transition and cell invasion of cancer cells in part through the small GTPase RAC1. Since RAC1 can signal through reactive oxygen species (ROS), we probed the role of the ROS-producing NADPH oxidase (NOX) and p38 mitogen-activated protein kinase (MAPK) in mediating TGF-β1/RAC1-driven random cell migration (chemokinesis). Although the NOX isoforms NOX2, 4, 5, 6, and RAC1 were readily detectable by RT-PCR in pancreatic ductal adenocarcinoma (PDAC)-derived Panc1 and Colo357 cells, only NOX4 and RAC1 were expressed at higher levels comparable to those in peripheral blood monocytes. TGF-β1 treatment resulted in upregulation of NOX4 (and NOX2) and rapid intracellular production of ROS. To analyze whether RAC1 functions through NOX and ROS to promote cell motility, we performed real-time cell migration assays with xCELLigence® technology in the presence of the ROS scavenger *N*-acetyl-L-cysteine (NAC) and various NOX inhibitors. NAC, the NOX4 inhibitor diphenylene iodonium or small interfering RNA (siRNA) to NOX4, and the NOX2 inhibitor apocynin all suppressed TGF-β1-induced chemokinesis of Panc1 and Colo357 cells as did various inhibitors of RAC1 used as control. In addition, we showed that blocking NOX4 or RAC1 function abrogated phosphorylation of p38 MAPK signaling by TGF-β1 and that inhibition of p38 MAPK reduced TGF-β1-induced random cell migration, while ectopic expression of a kinase-active version of the p38 activating kinase MKK6 was able to partially rescue the decline in migration after RAC1 inhibition. Our data suggest that TGF-β1-induced chemokinesis in PDAC cells is mediated through a RAC1/NOX4/ROS/p38 MAPK cascade.

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*Abbreviations:* DPI, diphenylene iodonium; EMT, epithelial-mesenchymal transition; MAPK, mitogen-activated protein kinase; NAC, *N*-acetyl-L-cysteine; qPCR, quantitative real-time RT-PCR; ROS, reactive oxygen species; TGF-β; transforming growth factor-β

*Key words:* TGF-β, NADPH oxidase, ROS, pancreatic ductal adenocarcinoma, NOX, RAC1, migration, p38 MAPK

## Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fifth leading cause of cancer-related mortality with ~70,000 estimated deaths each year and is predicted to become the fourth cause of cancer-related mortality in both sexes in due course in the European Union (1). This is in part caused by the biology of this tumor type which is characterized by early metastasis, poor response to chemotherapy and radiotherapy and overexpression of transforming growth factor (TGF)-β (2). This growth factor elicits its biological effects through type II and I transmembrane receptors with serine-threonine kinase activity and signaling intermediates from the Smad family of proteins, which transduce the TGF-β signal to the nucleus to alter the activity of responsive genes (3). TGF-β can also signal through several non-Smad pathways, e.g. PI3K/AKT, p38 mitogen-activated protein kinase (MAPK), small G-proteins such as RAC and RHO, and reactive oxygen species (ROS) (4,5). These Smad-independent TGF-β-induced pathways are thought to drive late events in cancer progression such as epithelial-mesenchymal transition (EMT), migration, invasion and metastasis (6). A better understanding of the signaling pathways involved in their induction by TGF-β may be mandatory to prevent malignant progression in PDAC.

Accumulating evidence suggests that ROS induce genetic instability and that cancer cells use redox signaling pathways to drive aberrant proliferation, survival and invasion, as well as interactions with the tumor microenvironment (reviewed in

ref. 7), eventually resulting in cancer progression (8,9). ROS are generated by cellular NADPH oxidases (NOX proteins) that catalyze the production of superoxide ($O^{2-}$) which serves as a starting material for the production of other reactive oxidants. The core enzyme from phagocytes comprises 5 components: $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, $p22^{phox}$ and $gp91^{phox}$ (=NOX2). Upon exposure to appropriate stimuli, the entire cytosolic complex (consisting of p40, p47 and p67) migrates to the membrane, where it associates with flavocytochrome b558 (consisting of $gp91^{phox}$ and $p22^{phox}$) to assemble the active oxidase (10).

Activation, in addition, requires the participation of RAC proteins (11) which are recruited to the membrane independently of $p47^{phox}$ or $p67^{phox}$. At the membrane, RAC is converted to the GTP-bound active form via the function of guanine nucleotide-releasing factors where it directly interacts with the $p67^{phox}$ N-terminal region, which is crucial for activation of $gp91^{phox}$. Binding of RAC is believed to induce a conformational change in $p67^{phox}$, which may allow the activation domain to act on $gp91^{phox}$/NOX2. Tumor cells harbor non-phagocytic NADPH oxidases that differ from the phagocyte NADPH oxidase in that they contain Rac1 rather than Rac2 as well as low-activity NOX2 homologs with modest levels of ROS formation (NOX1, NOX3-5, Duox1, Duox2) (10,12). NOX-derived ROS appear to be particularly important for activation of p38 and ERK MAPKs (13-15), a process which is thought to be mediated through the inactivation of various protein phosphatases (PP1, PP2A, MKP-1, PTP-1B) (16,17).

Our laboratory has previously demonstrated that TGF-β1 induces EMT in PDAC cells (18) along with upregulation of various mesenchymal markers such as the small leucine-rich proteoglycan biglycan (19). Notably, TGF-β1-induced biglycan expression was found to be dependent on activation of p38 MAPK which in turn required integrin ligation through adhesion (19) and both TGF-β1-induced biglycan expression and p38 MAPK activation required functional RAC1 and was sensitive to the NOX4/flavine oxidase inhibitor diphenylene iodonium (DPI) (20). Since conversion to a mesenchymal phenotype and integrin activation are considered prerequisites for epithelial cells to adopt a motile phenotype, it is conceivable that TGF-β-induced motility is coordinately controlled by RAC1, NOX and p38 MAPK signaling. Indeed, a previous study in a pancreatic cancer cell line showed that NOX4-derived ROS can transmit TGF-β-triggered EMT signals through PTP-1B (17). However, the role of RAC1 and p38 MAPK signaling in mediating the NOX4-derived ROS actions in response to TGF-β stimulation have not been analyzed. Specifically, a direct involvement of p38 MAPK, and its possible activation by RAC1 and NOX4, in TGF-β1-dependent random cell migration (chemokinesis) in PDAC-derived cells remains to be studied. In order to clarify this issue, several important questions must be addressed: i) are NOX isoforms expressed in response to TGF-β1 in PDAC-derived cells other than Panc1? ii) is the induction of chemokinesis by TGF-β1, ROS, RAC1, NOX4 and p38 MAPK-dependent? and iii) is p38 MAPK activation by TGF-β1, RAC1 and NOX4-dependent?

## Materials and methods

*Reagents*. The Nox2 inhibitor apocynin and the chemical antioxidant *N*-acetyl-L-cysteine (NAC) were purchased from Sigma (Deisenhofen, Germany). DPI, the p38 MAPK inhibitor SB203580, and the RAC1 inhibitor NSC23766 were obtained from Merck (Darmstadt, Germany). Recombinant human (rh) TGF-β1 was obtained from ReliaTech (Wolfenbüttel, Germany) and was used at a concentration of 5 ng/ml in all experiments.

*Ethical approval*. The present research does not consist of any studies using human participants or animals.

*Cell culture and treatments*. Human PDAC-derived Panc1 and Colo357 cells were cultured as previously described (18). The generation of Panc1 cells stably expressing a dominant-negative mutant of MKK6 (MKK6-Ala) was previously described (19). For the stimulation experiments, cells in normal growth medium [RPMI with 10% fetal calf serum (FCS)] were seeded into 6-well plates ($2x10^5$/well), 12-well plates ($0.8x10^5$/well) or 24-well plates ($0.4x10^5$/well) (all from Nunclon, Thermo Fisher Scientific, Dreieich, Germany) for the migration assays, protein analysis and qPCR, respectively. After cells had reached 80% confluence, they were starved overnight in medium containing 0.5% FCS. Cells were then treated with TGF-β1 for 24 h in the same medium in the presence or absence of the various inhibitors. These were administered to cells 30 min before the addition of TGF-β1.

*RNA isolation and RT-PCR*. Total RNA was isolated using PeqGold reagent (PeqGold, Erlangen, Germany) and further processed as recommended by the manufacturer. Synthesis of cDNA was carried out with M-MLV reverse transcriptase (Thermo Fisher Scientific). NOX protein subunit RNAs were either amplified with *Taq* polymerase (Life Technologies, Darmstadt, Germany) by standard endpoint PCR followed by agarose gel electrophoresis and ethidium bromide staining or by quantitative real-time RT-PCR (qPCR) on a CFX96 instrument (Bio-Rad, München, Germany) using SYBR-Green as previously described (18). The housekeeping gene TATA box-binding protein (TBP) was used for normalization. The human PCR primers used for amplification of NOX and PHOX mRNAs and TBP mRNA are listed in Table I.

*Immunoblotting*. Immunoblots were performed as described in detail previously (18,20). The antibodies used were anti-NOX4 (#14347-1-AP, Acris Antibodies), phospho-p38 MAPK (Thr180/Tyr182) (#4370), p38 MAPK (#3104), HSP90α/β (H-114, sc-7947) (all from Cell Signaling Technology, Frankfurt, Germany) and β-actin (Sigma). In some cases, a densitometric analysis of underexposed images was performed using the NIH ImageJ program.

*Transfection of small interfering RNAs (siRNAs) and expression vectors*. Panc1 cells were seeded in 12-well plates and transfected on the next day serum-free for 4 h with Lipofectamine RNAiMAX (Life Technologies) and 50 nM of either NOX4 (a mix of 4 premade siRNAs, ON-TARGETplus SMARTpool #L-010194-00-0005; Thermo Scientific) (for sequences see Table I), RAC1 siRNA (a mix of 4 premade siRNAs, siGENOME SMARTpool reagent, #M-003560), or a siCONTROL nontargeting siRNA (both from Dharmacon via Biomol, Hamburg, Germany). Following removal of the

Table I. PCR-primers and siRNAs.

| Name | Sequence (5'→3') |
|---|---|
| NOX1-forward | acaaattccagtgtgcagaccac |
| NOX1-reverse | agactggaatatcggtgacagca |
| NOX2-forward | gggctgttcaatgcttgtggct |
| NOX2-reverse | acatctttctcctcatcatggtgc |
| NOX3-forward | atgaacacctctggggtcagctga |
| NOX3-reverse | ggatcggagtcactcccttcgctg |
| NOX4-forward | ctcagcggaatcaatcagctgtg |
| NOX4-reverse | agaggaacacgacaatcagccttag |
| NOX5-forward | atcaagcggccccctttttttcac |
| NOX5-reverse | ctcattgtcacactcctcgacagc |
| $p22^{phox}$-forward | gtgtttgtgtgcctgctggagt |
| $p22^{phox}$-reverse | ctgggcggctgcttgatggt |
| $p47^{phox}$-forward | gtacccagccagcactatgtgt |
| $p47^{phox}$-reverse | aaagtagcctgtgacgtcgtct |
| $p67^{phox}$-forward | ttcgagggaaccagctgataga |
| $p67^{phox}$-reverse | gcatgggaacactgagcttcac |
| RAC1-forward | accatgcaggccatcaagtgtgtgg |
| RAC1-reverse | ttacaacagcaggcattttctcttc |
| β-actin-forward | gaccaggcccagagcaagag |
| β-actin-reverse | atctccttctgcttcctgtc |
| TBP-forward | gctggcccatagtgatcttt |
| TBP-reverse | cttcacacgccaagaaacag |
| NOX4 siRNA #1 | acuaugauaucuucuggua |
| NOX4 siRNA #2 | gaaauuaucccaagcugua |
| NOX4 siRNA #3 | gggcuaggauugugucuaa |
| NOX4 siRNA #4 | gaucacagccucuacauau |

transfection mixture, cells received standard culture medium and were allowed to recover overnight. Twenty-four hours after the first transfection, cells underwent a second round of transfection. In some experiments which comprised only one round of transfection, cells received Lipofectamine 2000 (Life Technologies) along with expression vectors for RAC1-N17, MKK6-EE (19) or empty vector (pcDNA3), alone or in combination. Forty-eight hours after the last transfection, cells were subjected to chemokinesis assay, immunoblot analysis, or qPCR.

*Real-time cell analysis (RTCA) migration assay*. Real-time cell analysis (RTCA) migration assays were performed with xCELLigence® technology (Acea Biosciences, San Diego, CA, USA, distributed by OLS, Bremen, Germany) as described elsewhere (18,21). In brief, experiments were performed according to the manufacturer's protocol with modified 16-well plates consisting of an upper and a lower chamber separated by a porous membrane (8-$\mu$m pores). The CIM-Plate 16 (OLS) is analogous to a Transwell plate, except that the underside of the membrane is equipped with microelectrodes for impedance-based detection of migrated cells. Prior to assembly, the underside of the porous membrane was coated with 30 $\mu$l of collagen I (Sigma) to facilitate adhesion of the cells and thereby increase the likelihood of cell-electrode contacts. To start an experiment, the lower chamber was filled with 165 $\mu$l of serum-reduced medium (containing 1% FCS plus inhibitors/TGF-β1) and the CIM-Plate 16 was placed in the RTCA DP device and incubated at 37˚C in 5% $CO_2$ for 1 h to equilibrate the medium followed by a measurement step for recording a background signal generated by cell-free media. Cells (30,000-60,000, overnight serum-starved, per well) were mixed and preincubated in 150 ml of culture medium containing 1% FCS and inhibitors/TGF-β1 at the same concentrations as in the lower chamber (chemokinesis setup). After 30 min, TGF-β1 was added and cells were seeded in the wells of the upper chambers. After cell addition, CIM-Plates 16 remained at room temperature in the laminar flow hood for 30 min to allow cells to settle onto the membrane. Phosphate-buffered saline (PBS) was added to the empty space surrounding the wells in order to prevent interference from evaporation. Each condition was performed in quadruplicate with a programmed signal detection (RTCA software version 1.2.1.; OLS) every 15 min for a total of 24-40 h, depending on the cell type and conditions.

*Measurement of ROS*. The generation of intracellular ROS was detected with $H_2$-DCF-DA (2',7'-dichlorodihydrofluorescein-diacetate; Molecular Probes, Karlsruhe, Germany) by flow cytometry. $H_2$-DCF-DA is first deacetylated to the non membrane-permeable $H_2$-DCF-DA and then oxidized, emitting a fluorescent signal. For the ROS assay, Panc1 cells were seeded in 6-well plates ($2x10^5$/well) with RPMI containing 10% FCS. When 80% confluent, the cells were incubated overnight in growth medium containing 0.5% FCS and on the next day were loaded with 10 mM $H_2$-DCF-DA for 30 min. Following this incubation, the medium was replaced with fresh medium containing TGF-β1 (5 ng/ml) and further incubated for various times. Following trypsinization, cells were centrifuged and resuspended in 500 $\mu$l PBS and fluorescence was measured by flow cytometry (FACS; BD Biosciences, Heidelberg, Germany) at a wavelength of 488 nm. For each sample, the specific fluorescence from 10,000 cells was determined using Cell Quest Software from BD Biosciences.

*Statistical analysis*. Statistical significance was calculated using the non-parametric Mann-Whitney U test. Data were considered significant at $p<0.05$.

## Results

*PDAC cells exhibit TGF-β1-inducible NOX4 expression and ROS production in response to TGF-β1 stimulation*. A study by Hiraga *et al* showed that NOX4-derived ROS signaling contributes to TGF-β-induced EMT in Panc1 cells (17). In order to test which NOX isoforms are expressed in PDAC-derived cells, we performed semi-quantitative RT-PCR analysis. Notably, Panc1 cells expressed NOX2, NOX4, NOX5, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and RAC1, while NOX1 and NOX3 were not detected, neither in the Panc1 cell line nor in freshly isolated peripheral blood monocytes used as control (Fig. 1A, left). A quantification of NOX2, NOX4, and NOX5 in Panc1 and Colo357 cells by qPCR revealed that NOX4 exhibited by far the strongest expression of all 3 isoforms with expression levels even higher than those

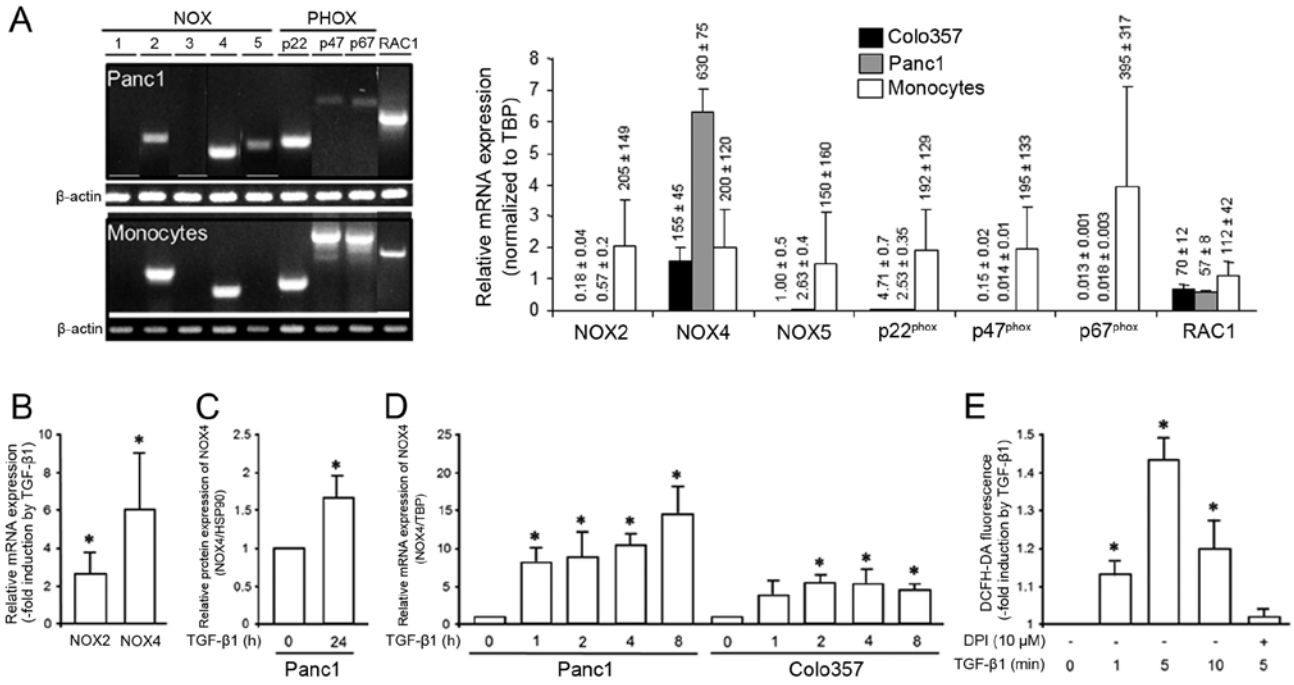


Figure 1. PDAC-derived cells upregulate NOX4 in response to TGF-β1 treatment and respond to TGF-β1 with DPI-sensitive ROS production. (A) Panc1 cells and freshly isolated peripheral blood monocytes were subjected to semi-quantitative RT-PCR with primers specific for NOX1-5, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and RAC1. The resulting amplification products were visualized by gel electrophoresis and ethidium bromide staining (left-hand side). On the right-hand side, results of qPCR analyses of NOX2, NOX4, NOX5, and RAC1 in Colo357 and Panc1 cells and in peripheral blood monocytes in relation to the housekeeping gene TBP are shown. (B) Colo357 cells were treated with TGF-β1 for 24 h and then subjected to qPCR for NOX2 and NOX4. Data represent the mean ± SD of 3 assays and are expressed as -fold induction by TGF-β1 over non-stimulated controls (n=3). (C) Panc1 cells were treated with TGF-β1 for 24 h and then subjected to immunoblot analysis for NOX4 and HSP90 as loading control. The graph shows results from densitometric analysis of band intensities from under-exposed blots (mean ± SD of 3 experiments). (D) As in C, except that cells were treated with TGF-β1 for shorter time periods (as indicated) and processed for qPCR rather than immunoblot analysis. Data shown are the mean ± SD of triplicate samples and are representative of 3 assays. (E) Panc1 cells were incubated with $H_2$-DCF-DA and remained untreated or were treated for the indicated times with TGF-β1, alone or in combination with DPI, and subsequently processed for detection of the oxidized dye by flow cytometry. Data represent the mean ± SD of triplicate samples. Asterisks in B-E indicate significance.

in peripheral blood monocytes (Fig. 1A, right). Panc1 cells have been shown to respond to TGF-β1 with upregulation of NOX4 (17). To ascertain whether NOX4, NOX2 and NOX5 are also induced in other PDAC-derived cells we treated Colo357 cells for 24 h with rhTGF-β1 and determined their expression by qPCR. These cells exhibited a strong upregulation of NOX4 (6.1±3.2, $p<0.05$), a moderate induction of NOX2 (2.5±1.1, $p<0.05$) (Fig. 1B), but no induction of NOX5 (data not shown). Due to its higher expression (see Fig. 1A) and the greater response to TGF-β1 (Fig. 1B) and the demonstration that it is a major source for ROS production in Panc1 cells (17) we focussed on NOX4 in all subsequent experiments. Upregulation of NOX4 by TGF-β1 was also noted at the protein level (Fig. 1C). To reveal whether the TGF-β1 effect on NOX4 temporarily correlates with the relatively rapid induction of cell migration by this growth factor (compare Fig. 3), we measured NOX4 expression in Panc1 and Colo357 cells after short stimulation periods. As shown in Fig. 1D, NOX4 expression was strongly induced already after 1 h of TGF-β1 treatment and remained elevated until 8 h after TGF-β1 addition.

Since PDAC cells appear to express the necessary components of the nonphagocytic enzyme, we tested more directly in Panc1 cells whether TGF-β1 was able to generate ROS. Intracellular ROS formation was monitored over a period of 20 min using $H_2$-DCF-DA, a redox-sensitive dye that readily diffuses into the cell where it is freed from the acetate groups by cellular esterases and converted into the highly fluorescent 2',7'-dichlorofluorescein in the presence of ROS. We observed a rapid, although moderate and transient increase in ROS peaking at 5 min (Fig. 1E). In the presence of the NOX4/flavine oxidase inhibitor DPI, TGF-β1-stimulated ROS production was suppressed (Fig. 1E). Although DPI is not specific for NOX4, high expression of this isoform and low or absent expression of the other NOX isoforms suggests that the observed effect was due to inhibition of NOX4. The data showed that TGF-β1 can induce ROS generation in PDAC-derived cells and, based on its high expression and its responsivity to TGF-β1 suggest that NOX4 accounted for TGF-β1-stimulated ROS production.

*NAC reduces TGF-β1-induced chemokinesis*. We previously showed in a series of studies that TGF-β1 is a powerful driver of random cell migration in PDAC-derived cells (18,21). To evaluate the possibility that TGF-β acts through intermediate production of ROS to control this response, we employed the antioxidant *N*-acetyl-cysteine (NAC). This drug which has been previously shown to be a specific inhibitor of TGF-β signaling (22) at 0.1-10 mM final concentrations strongly and in a dose-dependent manner downregulated the migratory response to TGF-β1 in both Panc1 and Colo357 cells (Fig. 2). Notably, in Panc1 cells, NAC also inhibited the early and

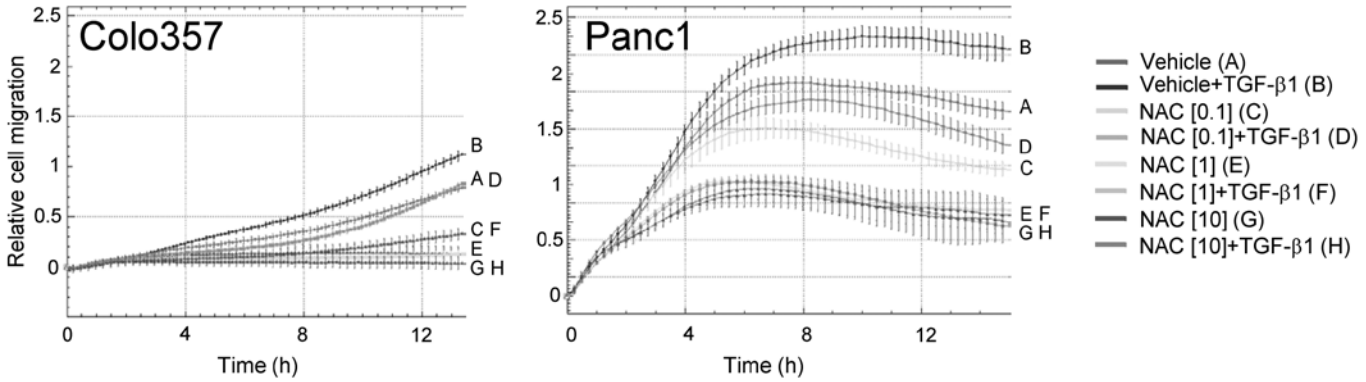


Figure 2. Effect of NAC on TGF-β-dependent chemokinesis. Colo357 and Panc1 cells were analyzed by real-time chemokinesis assay in the absence or presence of TGF-β1 and the indicated concentrations (in mM) of NAC or vehicle as indicated in the legends. Data are depicted as relative cell migration and are the mean ± SD of quadruplicate samples from a representative experiment out of three experiments performed in total. Letters to the right of each graph allow for color-independent identification of the various curves. For Colo357 cells, the inhibitory effect of 0.1 mM NAC on TGF-β1-treated vehicle control cells (tracing D vs. B) was significant at the 8 h and all later time points, the inhibitory effect of 1 mM NAC at the 5 h and all later time points (tracing F vs. B), and the inhibitory effect of 10 mM NAC at the 5 h and all later time points (tracing H vs. B). For Panc1 cells, the inhibitory effect of 0.1 mM NAC on TGF-β1-treated vehicle control cells was significant at the 5 h and all later time points, while the inhibitory effect of 1 mM and 10 mM NAC was significant at the 3 h and all later time points (tracing F and H, respectively, vs. B).

strong rise in migratory activity that was TGF-β1-independent up to ~4 h when the TGF-β effect initially became statistically significant (Fig. 2).

*Inhibition of NOX4 suppresses TGF-β1-induced chemokinesis*. Above we showed that Panc1 cells produced ROS in response to TGF-β1 and that the ROS scavenger NAC can blunt TGF-β1-mediated chemokinesis. TGF-β is known to produce ROS through the activation of RAC1 and we previously demonstrated that RAC1 is activated rapidly (within 5 min) by TGF-β1 in Panc1 cells (19). However, the above data led us to hypothesize that other components of the NADPH oxidase multi-subunit complex, e.g. NOX proteins are involved in mediating the TGF-β1 effect on random cell migration in PDAC cells. Above, we showed that expression of NOX4 was rapidly induced by TGF-β1 (within 1 h) in both Panc1 and Colo357 cells and was closely correlated temporarily with the TGF-β1 effect on cell migration. To verify the involvement of NOX proteins, in particular NOX4, we treated cells with DPI, which at 10 $\mu$M strongly inhibited TGF-β1-induced chemokinesis in Colo357 (Fig. 3A, left panel) and Panc1 (Fig. 3A, right panel) cells. Notably, the effect of DPI was generally more pronounced in Panc1 cells compared to Colo357 cells (Fig. 3A). As control, we employed the small molecule NSC23766 which blocks activation of RAC1 by the guanine nucleotide exchange factors TRIO and TIAM1. NSC23766 efficiently reduced TGF-β1-dependent chemokinesis in both Colo357 (Fig. 3B, left panel) and Panc1 (Fig. 3B, right panel) cells.

We complemented these data with a genetic approach using an siRNA to *NOX4*. Notably, NOX4 silencing in Panc1 cells by siRNA transfection led to a markedly reduced chemokinetic response to TGF-β1 (Fig. 3C, left panel). However, the effect was not as strong as that resulting from siRNA-mediated depletion of RAC1 which was performed as an internal control (Fig. 3C, left panel), or from ectopic expression of RAC1-N17, a RAC1 mutant that is unable to bind GTP and upon ectopic expression can block RAC1 activation in a dominant-negative fashion (Fig. 3C, right panel). As observed above for NAC, NSC23766 (Fig. 3B, right panel), DPI (Fig. 3A, right panel), RAC1-N17 (Fig. 3C, right panel) and NOX4 siRNA (Fig. 3C, left panel) also effectively inhibited the early, TGF-β1-independent phase of strongly increasing migratory activity (here, 0-7 h) in Panc1 cells. TGF-β1-induced chemokinesis was also inhibited by apocynin, a selective inhibitor of NOX2 (23) (data not shown). Together, these data clearly showed that both RAC1 and NOX proteins, particularly NOX4, play an essential role in the regulation of random cell migration by TGF-β1 and are in keeping with our assumption that RAC1 cooperates with NOX proteins to control TGF-β1-driven chemokinesis.

*Inhibition of p38 MAPK suppresses TGF-β1-induced chemokinesis*. The above results suggest that RAC1 and NOX4 are required for TGF-β1-induced chemokinesis of PDAC cells. Both RAC1 and ROS can activate p38 MAPK; however, whether p38 MAPK is also involved in TGF-β1-induced chemokinesis has not yet been demonstrated. To analyze this, we studied the effect of the p38 MAPK inhibitor SB203580 on TGF-β1-induced random cell migration of Colo357 and Panc1 cells in real-time cell migration assays. To this end, treatment of both cell types with SB203580 (10 $\mu$M) moderately and strongly, respectively, suppressed the chemokinetic response to TGF-β1 stimulation (Fig. 4A). Likewise, the migratory activity of Panc1 cells stably expressing MKK6-Ala, a dominant-negative inhibitor of MKK6-p38 MAPK signaling was reduced by 45% (p=0.00104) compared to the empty vector-expressing control cells (Fig. 4B). These data showed that the activation of p38 MAPK is crucial for TGF-β1-mediated chemokinesis and identified p38 MAPK as a downstream target of RAC1 and NOX4 in this process.

*Inhibition of NOX4 expression impairs TGF-β1-dependent activation of p38 MAPK*. To analyze whether inhibition of NOX4 expression/function impacts TGF-β1-induced p38 MAPK activation, we performed phospho-immunoblotting

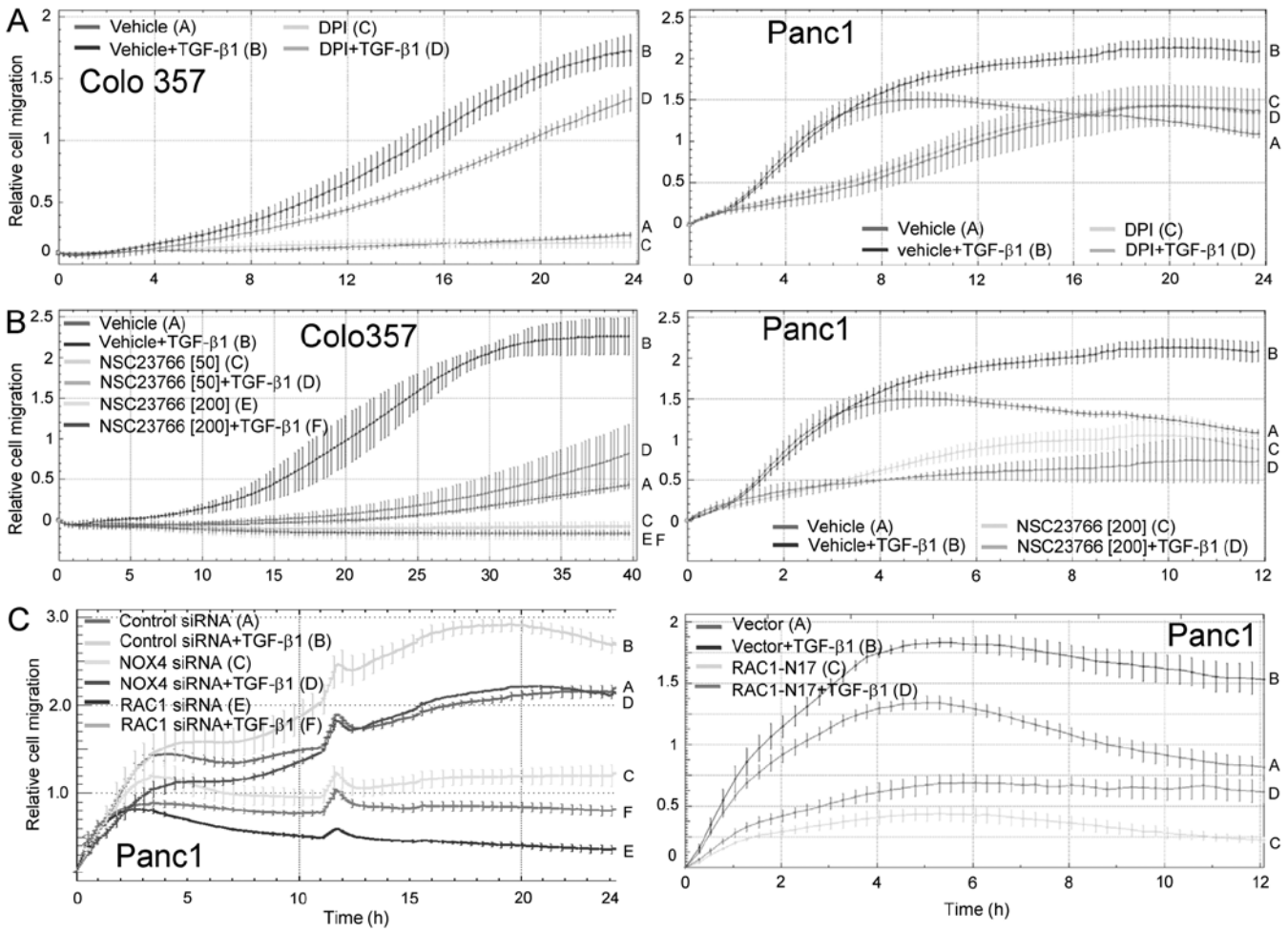


Figure 3. Inhibition of NOX4 expression suppresses TGF-β1-induced chemokinesis. Colo357 (A, left panel) and Panc1 (A, right panel) cells were stimulated with TGF-β1 in the absence or presence of the NOX4 inhibitor DPI followed by chemokinesis assay. The inhibitory effect of DPI on TGF-β1-dependent migration in Colo357 and Panc1 cells (tracing D vs. B) was first significant at 12 h and 3 h, respectively, and all later time points. As control, Colo357 (B, left panel) and Panc1 (B, right panel) cells were subjected to realtime chemokinesis assay in the presence of the chemical RAC1 inhibitor NSC23766 or vehicle as control. Data are depicted as relative cell migration and are the mean ± SD of quadruplicate samples of a representative experiment out of three experiments performed in total. For Colo357 cells, the inhibitory effect of TGF-β1 + NSC23766 (both 50 mM and 200 mM, tracing D and F, respectively) relative to TGF-β1 + vehicle control cells (tracing B) was first significant at the 10 h time point and remained so all later time points, while for Panc1 cells significance of the inhibitory effect for 200 mM NSC23766 + TGF-β1 was first noted at the 2 h time point (tracing D vs. B). (C, left panel) Panc1 were transfected twice by lipofection with irrelevant negative control siRNA, or siRNA to either NOX4, or siRNA to RAC1 as positive control for inhibition of TGF-β-dependent chemokinesis (see Fig. 5 for effectiveness of the NOX4 and RAC1 siRNAs in reducing expression of the respective proteins). Forty-eight hours after the second round of transfection, cells were subjected to realtime cell migration assay in the absence or presence of 5 ng/ml TGF-β1. The inhibitory effect of Nox4 siRNA and RAC1 siRNA on TGF-β1-dependent chemokinesis when compared to that of control siRNA transfected cells (NOX4: tracing D vs. B; RAC1: tracing F vs. B) was significant at 3 h and all later time points. (C, right panel) As an additional positive control, Panc1 cells were transiently transfected with RAC1-N17, an activation-deficient RAC1 mutant, or empty vector as negative control. Forty-eight hours after the start of transfection, cells were analyzed by realtime cell migration assay. Data are depicted as relative cell migration and are the mean ± SD of quadruplicate samples from a representative experiment out of three experiments performed in total. The inhibitory effect of TGF-β1-treated RAC1-N17 expressing cells relative to TGF-β1-treated empty vector control cells (tracing D vs. B) was first significant at 1 h and remained so at all later time points. Data in (A)-(C) are displayed as relative cell migration and are the mean ± SD of quadruplicate samples. In each panel, one representative experiment is shown out of three experiments performed in total.

with antibodies associated with p38 MAPK activation (Thr180/Tyr182). As shown in Fig. 5, transient transfection of NOX4 siRNA (Fig. 5, left panel), or RAC1 siRNA as control (Fig. 5, right panel), prevented the phosphorylation of these sites by TGF-β1. However, the expression of NOX4 was not affected by transfection of RAC1 siRNA (data not shown). These data showed that TGF-β1-driven activation of p38 MAPK is dependent on functional NOX4 and RAC1 protein. In addition, it is suggested that although NOX4 appears to function independently of RAC1, both proteins cooperate to induce p38 MAPK activation in order to mediate the TGF-β1 effect on chemokinesis.

*Ectopic expression of constitutively active MKK6 rescues cells from the RAC1-N17-mediated decline in TGF-β1-induced chemokinesis*. The data to date suggest that RAC1 and NOX4 act upstream of p38 MAPK in promoting cell migration. To prove that more directly, we ascertained whether activating p38 MAPK signaling, e.g. by ectopic expression of a kinase-active mutant of MKK6 (MKK6-EE), the upstream activator of p38, overcomes the antimigratory effect of Rac1 inhibition on TGF-β1-induced chemokinesis. To this end, while RAC1-N17 potently inhibited basal and TGF-β1-driven chemokinesis in Panc1 cells, co-transfection of MKK6-EE was able to partially rescue the cells from the decline in migratory activity (Fig. 6).

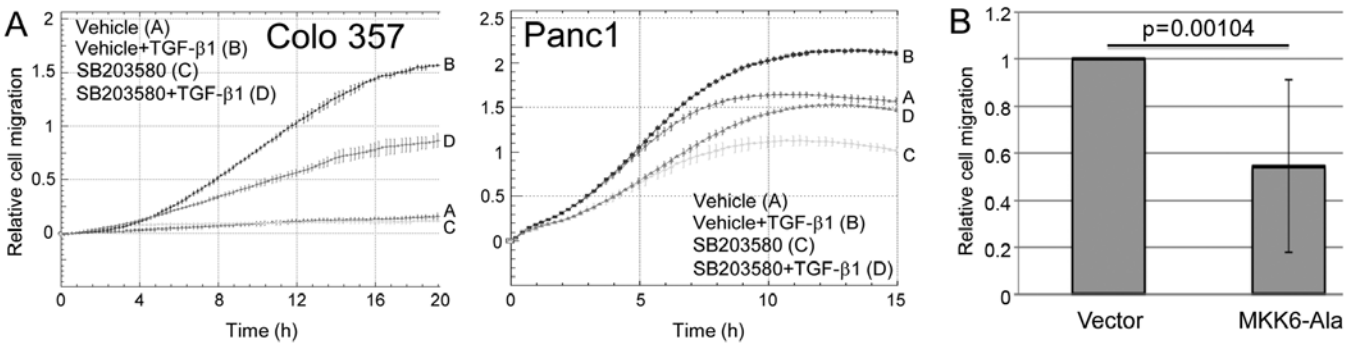


Figure 4. Effect of inhibition of p38 MAPK on TGF-β1-induced chemokinesis. (A) Colo357 and Panc1 cells, treated or not with TGF-β1, were analyzed by migration assay in the absence or presence of SB203580 (10 $\mu$M). Data are depicted as relative cell migration and are the mean ± SD of triplicate samples. For each cell line, a representative experiment is shown out of three experiments performed in total. For Colo357 and Panc1 cells, the inhibitory effect of TGF-β1 + SB203580 treated cells (tracings D) relative to TGF-β1 + vehicle treated control cells (tracings B) was significant at 8 h and 4 h, respectively, and at all later time points. (B) Quantitative analysis of chemokinesis assays performed with Panc1 cells stably expressing MKK6-Ala or empty vector as control. Data (mean ± SD, n=3) are depicted as relative chemokinetic activity of TGF-β1-treated MKK6-Ala expressing cells over TGF-β1-treated empty vector expressing cells with the vector control cells set arbitrarily at 1 in each of the three experiments.

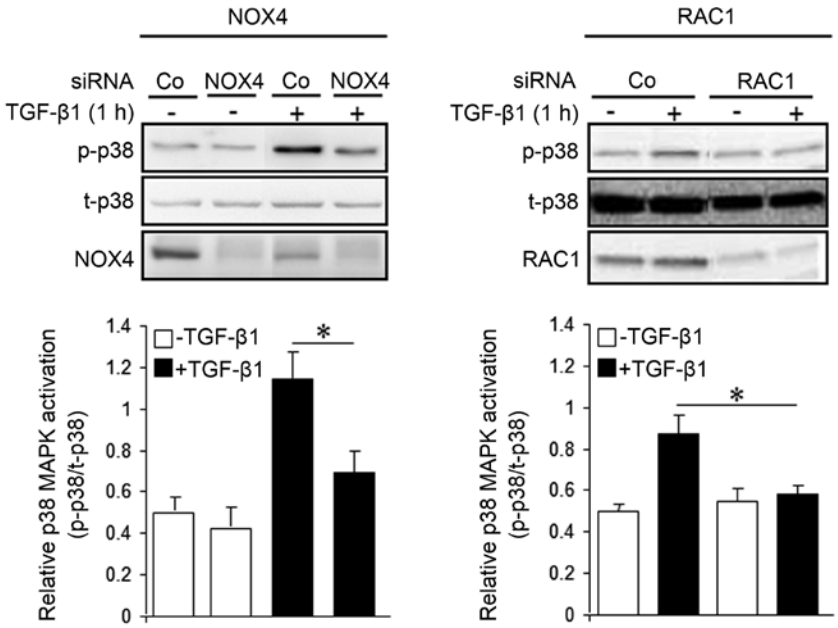


Figure 5. Cellular depletion of NOX4 by RNAi impairs TGF-β1-dependent activation of p38 MAPK. Panc1 cells were transfected twice with either NOX4 siRNA or scrambled control siRNA (left panel), or siRNA to RAC1 or an irrelevant control siRNA (right panel). Forty-eight hours after the second round of transfection, cells were treated with TGF-β1 for 1 h and subjected to immunoblotting for phospho-p38 MAPK (p-p38), total p38 MAPK (t-p38), and NOX4 or RAC1, respectively. The graphs underneath the blots show the results from densitometric analysis of underexposed blots with band intensities plotted as ratios of p-p38 over t-p38. Each blot is representative of 3 assays. The asterisks indicate significance.

These data provide additional evidence for our contention that p38 MAPK acts downstream of RAC1 and NOX4 in promoting TGF-β1-dependent chemokinesis.

## Discussion

Previous studies from our group have shown a crucial role for RAC1 in TGF-β/Smad and non-Smad-mediated cellular responses. For instance, functional RAC1 was necessary for TGF-β-dependent Smad2 C-terminal phosphorylation and induction of biglycan gene expression as demonstrated by ectopic expression of RAC1-N17 in Panc1 cells (18). In the present study, we showed that activated RAC1 was crucial for mediating the pro-migratory effect of TGF-β1 since both the small molecule Rac1 inhibitor NSC23766 and RAC1-specific siRNA, and RAC1-N17 were able to inhibit TGF-β1-driven random cell migration.

Since RAC1 constitutes a subunit of the NOX multienzyme complex, we investigated the possibility that TGF-β1-activated RAC1 acts through NOX4-mediated production of ROS to drive chemokinesis. A combination of semi-quantitative RT-PCR and qPCR analyses revealed that although PDAC-derived Colo357 and Panc1 cells expressed several NOX isoforms, only NOX4 displayed expression levels in the range of those in peripheral blood monocytes. Moreover, NOX4 mRNA was potently induced by a 24-h treatment with TGF-β1 in Colo357 cells (6.1±3.2-fold, $p<0.05$), together suggesting NOX4 as the NOX isoform required for TGF-β1-dependent

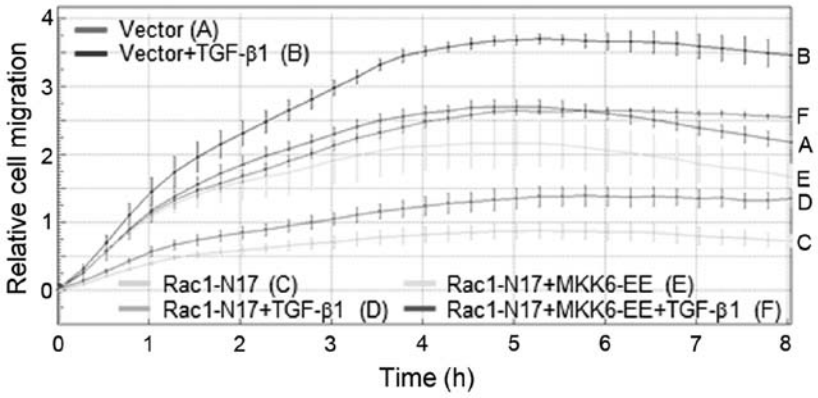


Figure 6. Ectopic expression of MKK6-EE rescues Panc1 cells from the inhibitory effect of RAC1-N17 on TGF-β1-dependent cell migration. Panc1 cells were transfected with equal amounts of either empty vector, an expression vector for RAC1-N17, or a combination of RAC1-N17 and MKK6-EE. Forty-eight hours later, cells were processed for migration assay in the absence or presence of TGF-β1. Data are depicted as relative cell migration and are the mean ± SD of triplicate samples of a representative experiment out of three experiments performed in total. The rescue effect of TGF-β1-treated RAC1-N17+MKK6-EE expressing cells relative to TGF-β1-treated RAC1-N17 expressing cells (tracing F vs. D) was first significant at 1 h and remained so at all later time points.

cell migration. In line with this, Panc1 cells responded to TGF-β1 treatment with a rapid and time-dependent generation of ROS that was sensitive to the NOX4 inhibitor DPI. The fast kinetics of ROS production in response to TGF-β1 stimulation which parallels that of NADPH oxidase activity (14) is in good agreement with the rapid activation of RAC1 in these cells (<5 min) (20) and its rapid translocation (within 5 min) from the cytoplasm to the membrane (14). Both events precede the activation of p38 MAPK (20) which is first detectable at 10-15 min and peaks after 1 h of TGF-β1 treatment (14,24). The activated p38 MAPK then drives cell migration as the time course of p38 MAPK activation parallels that of the migratory response (19). In line with a role of ROS in TGF-β1-dependent cell motility, treatment of Panc1 and Colo357 cells with the radical scavenger NAC suppressed TGF-β1-mediated chemokinesis in a dose-dependent manner.

The hypothesis that NOX4 drives TGF-β1-dependent chemokinesis through ROS was given further credit by the results from inhibition experiments with DPI and an NOX4-specific siRNA both of which were able to inhibit TGF-β1-driven random cell migration. Hiraga *et al* previously showed that NOX4 expression was induced by TGF-β1, was upregulated in tumors from PDAC patients and was required for TGF-β1-induced ROS production. Using Boyden chamber assays in combination with a chemotaxis setup, these authors showed that NOX4 was involved in TGF-β regulation of directed cell migration/chemotaxis (17). We extended these data by demonstrating that NOX4 is also involved in random cell migration/chemokinesis induced by TGF-β1. Moreover, by employing real-time rather than endpoint measurements, we revealed that in Panc1 cells NAC or DPI treatment, or NOX4 or RAC1 depletion also inhibited the initial sharp rise in migratory activity that was independent of TGF-β1. This rapid anti-migratory effect was likely mediated by inhibition of SRC as we have shown previously that the initial, TGF-β-independent rise in migration was SRC-dependent (25). In line with this, TGF-β1-initated c-SRC-Y416 activation is known to be ROS-dependent (26). These findings remained undiscovered by Hiraga *et al* since their Boyden chamber assays only allowed for endpoint measurements. It should be mentioned that NOX4 has also been implicated in TGF-β-driven EMT and migration of breast epithelial cells (27), and in the stimulation of breast cancer cells by mammary cells of stromal origin (28).

Vaquero *et al* were the first to demonstrate that a nonmitochondrial NADPH oxidase is a major source of growth factor-induced ROS in pancreatic cancer cells (29), and that these cells express various subunits and NOX isoforms of the phagocytic NADPH oxidase including phagocytic NOX2. Notably, we found that NOX2 inhibition with the prodrug apocynin in Panc1 and Colo357 cells reduced the TGF-β effect on chemokinesis (data not shown). It may be interesting to test the idea that NOX2 acts in concert with NOX4 which could help to explain why inhibiting NOX4 was not as effective as inhibiting RAC1 in suppressing TGF-β1-induced chemokinesis (Fig. 3C). However, definite conclusions should not be based on the use of these inhibitors alone as their specificity has been questioned (30).

We further evaluated the possibility that TGF-β1-induced NOX4 protein drives random migration through p38 MAPK signaling. This assumption was supported in the present study by the demonstration that blocking p38 MAPK by pharmacological inhibition with SB203580 abrogated TGF-β1-induced chemokinesis. Moreover, treatment with the NOX4 inhibitor DPI (20) or NOX4 depletion by RNAi (Fig. 5) prevented p38 MAPK activation by TGF-β1 in Panc1 cells, identifying p38 MAPK as a mediator of NOX4-dependent TGF-β1-driven chemokinesis. Again, NOX4 functionally cooperates with RAC1 through ROS production since both siRNA-mediated depletion of RAC1 (the present study, Fig. 5) and stable ectopic expression of RAC1-N17 (ref. 20 and the present study, Fig. 3C, right panel) was able to mimic the NOX4 effect on TGF-β1-induced p38 MAPK activation (Fig. 5) and chemokinesis (Fig. 3). However, NOX4 does not appear to be functionally linked to RAC1 since depleting RAC1 protein from Panc1 cells did not affect the expression of NOX4, an important observation that is in line with data from other studies (31-33, reviewed in ref. 34).

Enhanced ROS production as observed in inflammation and cancer can cause severe tissue damage. TGF-β1 via RAC1, NOX4, ROS intermediates and p38 MAPK promote not only cell migration/invasion and metastasis but also the synthesis

and accumulation of extracellular matrix proteins such as biglycan. Thereby, TGF-β1 favors the development of fibrosis and desmoplasia, the latter being a hallmark feature of PDAC. Antioxidants, radical scavengers, or RAC1/NOX(4)/p38 MAPK inhibitors that can abrogate TGF-β signaling may thus be promising tools with which to prevent desmoplasia, migration, invasion and metastasis.

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